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Cloning and sequence analysis of canine apoptosis-associated molecules

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Für
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Abbreviations

aa	amino acid
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BCL2L	Bcl-2-like (e.g. BCL2L1 = Bcl-XL)
BH domain	Bcl-2 Homology domain
Bid	BH3 interacting domain death agonist
BIR	Baculovirus Inhibitor of Apoptosis Repeat
BIRC	Baculovirus Inhibitor of Apoptosis Repeat containing (e.g. BIRC5 = Survivin)
Bmf	Bcl-2 modifying factor
Bok	Bcl-2-related ovarian killer protein
bp	Base pairs
CARD	Caspase Recruitment Domain
cDNA	complementary DNA
cds	coding sequence
cIAP-1/-2	cellular Inhibitor of Apoptosis Protein 1/2
DED	Death Effector Domain
DNA	Deoxyribonucleic Acid
ESTs	Expressed Sequence Tags
HtrA2	High temperature requirement protein A2
IAPs	Inhibitors of Apoptosis Proteins
IBM	IAP Binding Motif
MDCK cells	Madin-Darby Canine Kidney cells
ML-IAP	Melanoma Inhibitor of Apoptosis Protein
mRNA	messenger RNA
NAIP	Neuronal Apoptosis Inhibitory Protein
NCBI	National Center for Biotechnology Information
PMAIP1	Phorbol-12-myristate-13-acetate-Induced Protein 1 (Noxa)
RNA	Ribonucleic Acid

RT-PCR	Reverse Transcription Polymerase Chain Reaction
Smac	Second mitochondria-derived activator of caspase
TAF-1	TBP-Associated Factor 1
XIAP	X-linked Inhibitor of Apoptosis Protein

1. Summary

The aim of the study was to clone and sequence the coding sequences of a representative set of proteins, primarily from the intrinsic apoptotic pathway in dogs and to assess their conservation with human and murine orthologues.

cDNA for these proteins, including Bcl-2 family members (Bcl-XL, Bcl-w, Mcl-1, Bax, Bak, Bad, Noxa), caspases (Caspase-3, Caspase-8, Caspase-9), Inhibitors of Apoptosis Proteins (XIAP, cIAP-1, cIAP-2, Survivin), their mitochondrial inhibitors (Smac/ DIABLO, Omi/HtrA2) and p53, were generated by RT-PCR with RNA extracted from two canine non-neoplastic cell lines. Eleven sequences are novel for the dog.

Interspecies comparison revealed strongest similarity between the sequences of human and canine intrinsic apoptosis pathway members. Differences with potential functional impact, however, were observed in both dogs and mice. In dogs, these changes involve the putative Inhibitor of Apoptosis Protein binding motif of canine Omi/HtrA2, some caspase substrate recognition motifs and some functionally relevant residues of p53. Canine XIAP yields a caspase-cleavage site reported as unique to humans.

In conclusion, the generally high degree of similarity of canine apoptosis-associated proteins as compared to human counterparts is supportive of the use of dogs as a model for human diseases. Single interspecies sequence variations with potential functional relevance under physiologic and neoplastic conditions do exist, however, and will require further analysis.

2. Zusammenfassung

Ziel der vorliegenden Arbeit war es, eine repräsentative Gruppe von Molekülen, die hauptsächlich am intrinsischen Signalweg der Apoptose beteiligt sind, zu klonieren und sequenzieren, und mit den Homologen von Mensch und Maus zu vergleichen.
Mittels RT-PCR, basierend auf RNA aus zwei kaninen nicht-neoplastischen Zelllinien, wurden cDNAs von Bcl-2 Familienmitglieder (Bcl-XL, Bcl-w, Mcl-1, Bax, Bak, Bad, Noxa), Kaspasen (Kaspase-8, -9, -3), "Inhibitors of Apoptosis" Proteinen (XIAP, cIAP-1, cIAP-2, Survivin), Smac/DIABLO, Omi/HtrA2 und p53 generiert. Elf dieser Sequenzen werden beim Hund erstmals beschrieben.
Die höchste Konservierung der Nukleotid- und Proteinsequenzen wurde zwischen Hund und Mensch festgestellt. Sequenzunterschiede zum Menschen mit potentiell funktionellem Einfluss wurden bei Hund und Maus beobachtet. Darunter fallen beim Hund das mutmassliche "Inhibitor of Apoptosis Protein Binding Motif" von Omi/HtrA2, einige Substraterkennungsmotive von Kaspasen, sowie einige funktionell relevante Aminosäuren von p53. Des weiteren weist das kanine XIAP eine Kaspasen-Spaltstelle auf, die bisher als einzigartig für den Menschen galt.
Die generell hohe Ähnlichkeit der Apoptose-assoziierten Proteine des Hundes mit denen des Menschen befürwortet dessen Einsatz als Modelltier für humane Erkrankungen. Einzelne speziesspezifische Sequenzunterschiede mit potentieller funktioneller Relevanz unter physiologischen und neoplastischen Bedingungen bedürfen jedoch weiterer Untersuchungen.

3. Introduction and aim of the study

Apoptosis is a form of cell death resulting from the activation of a genetically determined cell suicide program. It is essential for proper development and tissue homeostasis, and it plays an important role in host defense mechanisms. DNA-damage is an important trigger of apoptosis, which elicits the elimination of cells with irreparable genetic defects and therefore constitutes an essential protection mechanism against cancer development (Cory et al. 2002; Cory et al. 2003). Conversely, deregulated apoptosis is a hallmark of cancer (Hanahan et al. 2000) and plays roles in tumorigenesis and in the development of resistance to therapy (Cory et al. 2002; Johnstone et al. 2002).

Spontaneous cancer is the most common cause of death in dogs (Vail et al. 2000). Dogs develop a range of spontaneous neoplasms often with a histology and biologic behavior similar to human tumor diseases, including non-Hodgkin's lymphoma, soft-tissue sarcoma, osteosarcoma, and metastasis (e.g. to bone) (Vail et al. 2000; Rosol et al. 2003; Khanna et al. 2004). Because of their great significance as companion animals, canine tumor patients are increasingly subjected to therapy and hence provide a potential resource for elucidating pathogenesis and modeling therapy-related aspects of human cancer. However, the general knowledge pertaining to canine molecules involved in apoptosis, as pointed out in more detail further below, is scant. In addition, despite the relatively limited number of known canine sequences, a few sequence differences suggesting the existence, at least from a mechanistical point of view, of differences in the pathway function have been reported and will be addressed further below. Awareness of such variations is important. On the one side, they possibly impinge on the pathway function under physiological and/or neoplastic conditions, which ultimately could generate significant interspecies differences in the mechanisms involved in tumorigenesis. On the other hand, the finding of interspecies differences in individual motifs potentially helps in the assessment of their relative importance.

The aim of the present work was to extend present knowledge on the molecules involved in the intrinsic apoptotic pathway in the dog by cloning and sequencing a representative set of molecules belonging to this group. The complete coding sequences of cDNAs for some of the most important canine members of the Bcl-2 family, the Caspases, the IAP family, their mitochondrial inhibitors Omi/HtrA2 and

Smac/DIABLO as well as p53 were determined. The degree of homology of the canine molecules with the human and murine counterparts was compared both at the full-length level (nucleotides of the coding sequences and amino acid sequences) and for selected specific domains and motifs.

4. Overview of Apoptosis with special emphasis on the intrinsic apoptotic pathway

Since the first description of apoptosis 35 years ago as a form of cell death with a characteristic morphology (Kerr et al. 1972), the underlying biochemical mechanisms and the molecules involved have been quite extensively characterized. At present, the two main apoptotic pathways commonly referred to are the extrinsic and the intrinsic apoptotic pathway. The extrinsic pathway is triggered by the engagement of cell surface death receptors by cognate ligands, which results in trimerization of the receptors, recruitment of specialized adaptor proteins and initiator caspases, and their activation. This part is excluded from the present work, which focuses on the intrinsic pathway.

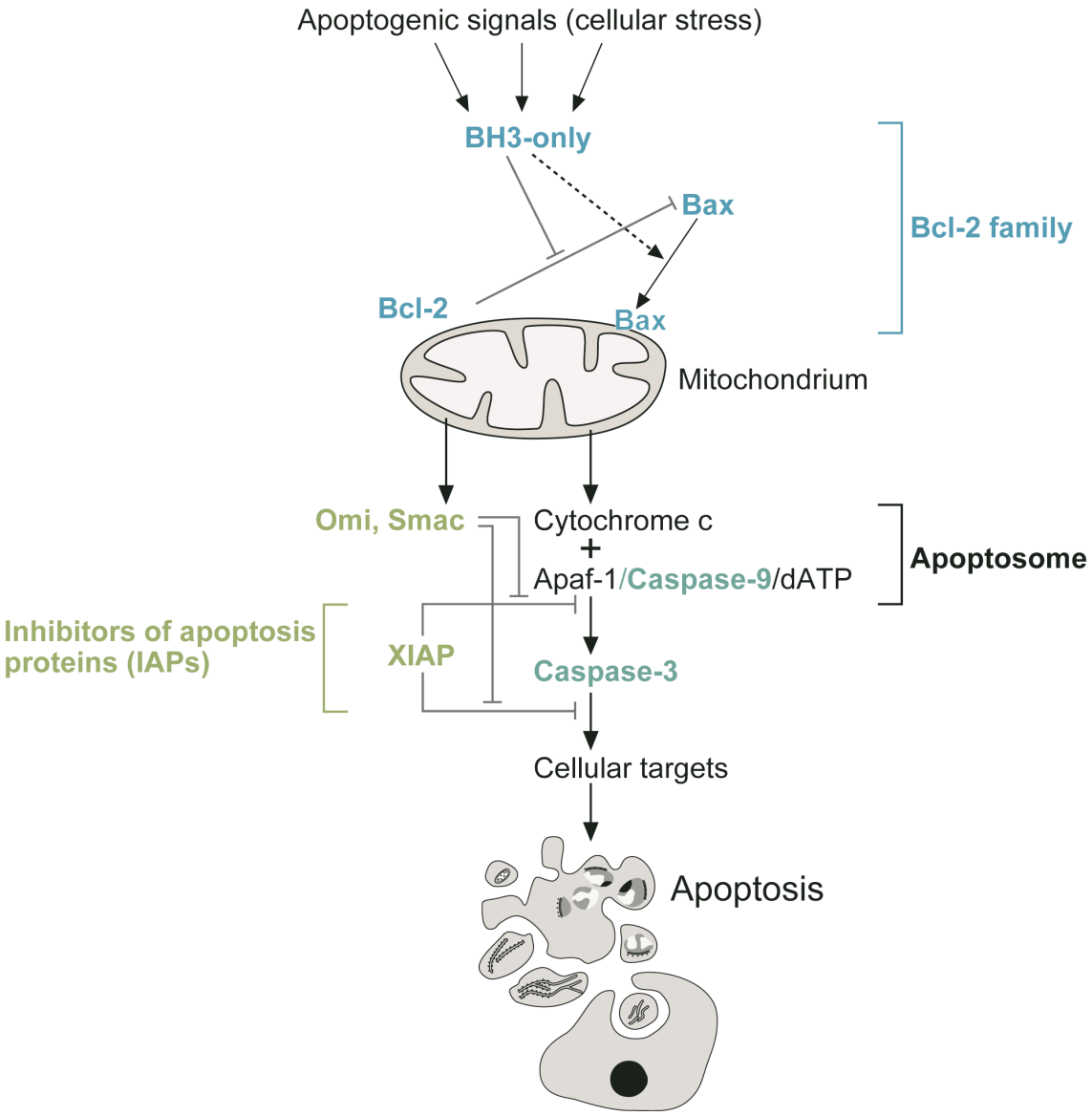
This pathway is engaged following conditions collectively termed as "cellular stress" and including a number of different stimuli such as genome damage, oncogene activation, and others. A schematic depiction of this pathway is presented in Figure 1. The key event is characterized by a permeabilization of the outer mitochondrial membrane, a step controlled by molecules of the Bcl-2 family, and which is associated with the release of apoptogenic factors including cytochrome c into the cytosol (Cory et al. 2002). In the presence of ATP, cytoplasmic cytochrome c leads to the assembly of an oligomeric complex, named apoptosome, which contains several copies of an adaptor molecule (Apaf-1) and two Caspase-9 molecules. Within this complex Caspase-9 is activated and it initiates a proteolytic cascade resulting in apoptosis (Shi 2002).

While the bulk of the present knowledge on apoptotic pathways derives from studies on human and murine cells, a significant portion of knowledge originates from studies with simpler organisms, in particular *Caenorhabditis elegans* and, to a lesser extent, *Drosophila melanogaster* (Stergiou et al. 2004). In contrast, comparatively little information is available on apoptosis-associated molecules in dogs. A small number of these have been cloned and characterized, including the complete coding sequences for

Bcl-2, Bcl-XL, Mcl-1, Bax, Survivin, Caspase-3 and p53, as well as incomplete coding sequences of a few further molecules (Veldhoen et al. 1998; Sano et al. 2003; Sano et al. 2004; Sano et al. 2004; Uchide et al. 2005). There is anecdotal evidence of differences at the molecular level within the apoptotic pathway between humans and dogs. As an example, canine and murine Caspase-9 lack a consensus Akt phosphorylation site proposed to act by preventing the activation of this protease in humans (Cardone et al. 1998; Rodriguez et al. 2000). Another example is a minor caspase substrate recognition motif within the aminoacid sequence of the Bcl-2 family member Mcl-1 which is not conserved in the dog as compared to humans (Michels et al. 2004). These examples call for an extended search for further interspecies differences.

In the following an overview is given on selected protein families involved in the intrinsic apoptotic pathway with a brief description of their principal domains and mechanisms of function as far as necessary for the understanding of the following analysis.

Figure 1. Schematic depiction of the intrinsic apoptotic pathway



Bcl-2 = prototype for anti-apoptotic Bcl-2 family members; Bax = prototype for multi-domain pro-apoptotic Bcl-2 family members; Caspase-9 = initiator caspase; Caspase-3 = prototype for effector caspases; XIAP = prototype for Inhibitor of Apoptosis Proteins (IAPs).

The Bcl-2 family

Proteins are assigned to the Bcl-2 family owing to the presence of at least one of four possible BH domains. Their relative occurrence provides support to further subdivide the family into three subgroups (Cory et al. 2002). The anti-apoptotic members include Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1. They possess all four BH domains with the exception of Mcl-1, which has no BH4 domain (Cory et al. 2003). The second subgroup consists of so-called multi-domain pro-apoptotic members and include Bax, Bak and Bok. They display domains BH1 to BH3. Bax and Bak are required for the execution of apoptosis through the mitochondrial pathway (Wei et al. 2001). Members of the third subgroup, the BH3-only molecules, possess a BH3 domain only. This subgroup is composed of a large number of members including Bad, Noxa, Bid, Bim, Bmf and several others (Cory et al. 2003). Activation of BH3-only molecules upon appropriate stimuli perturbs the balance between anti-apoptotic and multi-domain pro-apoptotic members characteristic for non-apoptotic cells. As a result, the multi-domain pro-apoptotic molecules can oligomerize and form channels in the outer mitochondrial membrane allowing release of apoptogenic factors (e.g. cytochrome c) from the intermembrane mitochondrial space into the cytosol (Cory et al. 2002). BH3-only molecules are activated through diverse mechanisms. For example, Noxa is regulated at the transcriptional level (it is a target of p53), while Bad is maintained in an inactive state in the cell cytoplasm through phosphorylation of several serine residues which mediates binding to cytosolic 14-3-3Sigma proteins. The protein is dephosphorylated upon pro-apoptotic signals and subsequently it interacts with anti-apoptotic members (Bae et al. 2001).

The BH domains mediate the interplay between the family members which control the integrity of the outer mitochondrial membrane. Structural studies have shown that in anti-apoptotic molecules such as Bcl-XL specific residues of different domains (i. e. BH1, BH2 and BH3) participate to form a hydrophobic groove which interacts with the amphipathic helix of the BH3 domain of molecules of the other subgroups (Cory et al. 2002). Several recent studies have demonstrated variable affinities between the BH3 domains of individual BH3-only members and anti-apoptotic molecules (Chen et al. 2005; Kuwana et al. 2005).

Although the Bcl-2 family members altogether participate to the control of the permeability of the outer mitochondrial membrane, under physiologic conditions some

members, as e. g. Bax, or as indicated above, Bad, are located extra mitochondrially. Several anti-apoptotic and multi-domain pro-apoptotic members of the Bcl-2 family display a transmembrane domain which targets the molecules mostly to the outer mitochondrial membrane (Cory et al. 2003).

Caspases and specific substrate recognition motifs

Caspases are proteolytic aspartate-specific cysteine proteases present as proenzymes in the cell cytoplasm and are activated in a cascade-like manner (Shi 2002). Pro-apoptotic stimuli elicit the activation of (apical) initiator caspases which can undergo autocatalysis and which cleave and activate the (distal) effector caspases. Activated effector caspases consist of homodimers. They cleave specific cellular substrates, leading to apoptosis (Shi 2002).

Caspases involved in the intrinsic apoptotic pathway include Caspase-9 and the downstream Caspase-3, Caspase-7 and Caspase-6. The proenzymes consist of a pro-domain and two subunits (one large and one small) released by cleavage after specific aspartate residues (Nicholson 1999). In human Caspase-9, the subunits are connected by a linker (Shi 2002). The four residues immediately preceding each cleavage site constitute a specific substrate recognition motif (Nicholson 1999). The active site of the caspases is located at the C-terminus of the large subunit (Thornberry et al. 1997).

Initiator caspases are predicted to be activated through a "proximity-induced dimerization" mechanism (Shi 2004). An example of this mechanism is the adaptor molecule-mediated dimerization of Caspase-9 molecules, which are brought into close proximity in the apoptosome and subsequently undergo conformational changes leading to the formation of one functional active site. This implies that cleavage is not necessary for activation of Caspase-9 (Stennicke et al. 1999). In addition, Procaspase-9 can be cleaved by Caspase-8, one of the initiator caspases central to the extrinsic apoptotic pathway, thus providing one of several links between the two pathways (McDonnell et al. 2003).

Interestingly, a relatively large number of apoptosis-associated molecules contain Caspase-3 substrate recognition motifs. Cleavage has been shown in most instances either to convert the function of these proteins from anti-apoptotic to pro-apoptotic, or to enhance their pro-apoptotic activity, thus amplifying apoptosis in the final stage (Kirsch et al. 1999; Herrant et al. 2004; Fan et al. 2005).

Inhibitors of Apoptosis Proteins (IAPs)

The eight members of this family of evolutionarily conserved proteins (XIAP, cIAP-1, cIAP-2, Survivin, NAIP, Apollon, ML-IAP, and Ilp-2) exert their anti-apoptotic function to a large part by binding to and inhibiting activated caspases (Vaux et al. 2003). Proteins are assigned to the IAP family based on the presence of a Baculovirus Inhibitor of Apoptosis Repeat (BIR) domain, which is a zinc-binding fold associated with binding and inhibition of caspases. The anti-apoptotic members cIAP-1, cIAP-2 and XIAP each contain three distinct BIR (BIR1 to BIR3) domains some of which mediate binding to the caspases. However, the functions of the BIR domains appear to be diverse and have not been fully elucidated in each case. The BIR3 of human XIAP, one of the best characterized IAPs, binds the IAP binding motif (IBM) of Caspase-9 through a surface groove, thus locking Caspase-9 in its inactive monomeric state (Shiozaki et al. 2003). The BIR2 domain of human XIAP binds Met^{182} of Caspase-3 by docking it into a hydrophobic pocket formed by Tyr^{154} and Phe^{228} (Riedl et al. 2001). Recent data indicate that this binding does not result in inhibition of Caspase-3 (Scott et al. 2005). Inhibition of Caspase-3 and -7 has been shown to be associated with binding of the linker residues N-terminal to the BIR2 (human $DISD^{151}$) of XIAP to the active site of the caspases, preventing substrate binding (Riedl et al. 2001). Recently, the BIR2 and BIR3 domains of cIAP-1 and cIAP -2 have been shown to bind Caspase-7 and -9 without inhibiting the caspase function (Eckelman et al. 2006). The BIR1 domain of XIAP interacts with Tumor Necrosis Factor (TNF) Receptor-associated Factor 2, thus providing a link to the extrinsic apoptotic pathway (Samuel et al. 2006).

In addition, cIAP-1, cIAP-2 and XIAP contain a RING-type motif, which is required for auto- or trans-ubiquitination and regulation of the IAP levels in the cell (Silke et al. 2005). Finally, cIAP-1 and cIAP-2 display a Caspase Recruitment Domain (CARD), whose function is still not completely understood.

Survivin is a bifunctional IAP with an established function in the regulation of mitosis and a somewhat less-well characterized anti-apoptotic function. It contains a single BIR domain which shows close similarity to the BIR3 domain of XIAP (Shi 2000; Sun et al. 2005).

Furthermore it has been suggested that Survivin interacts with, and inhibits, Caspase-9 and structural studies have characterized its binding interface with Smac/DIABLO

(O'Connor et al. 2000; Sun et al. 2005). This binding occurs in a similar way as between the BIR3 of XIAP and Smac/DIABLO but with a lower affinity (Sun et al. 2005).

Mitochondrial inhibitors of IAPs

The nuclear-encoded mitochondrial proteins Smac/DIABLO and Omi/HtrA2 are released from the mitochondrial intermembrane space into the cell cytoplasm upon appropriate pro-apoptotic stimulation. Initially, both proteins are directed as pre-proteins to the mitochondria through classical targeting sequences located at the N-terminus and removed following entry into this organelle. The ensuing mature proteins display a N-terminal IBM motif. The IBM motif mediates binding of IAPs, which results in release of bound caspases as demonstrated for XIAP and other IAPs (Suzuki et al. 2001; Martins et al. 2002; van Loo et al. 2002) (Verhagen et al. 2000; Srinivasula et al. 2003; Bartke et al. 2004; Suzuki et al. 2004; Sekine et al. 2005), but, in the case of Omi/HtrA2, not for Survivin (Verhagen et al. 2002), abrogating the inhibitory effect of the IAPs on the caspases. IBMs are phylogenetically highly conserved down through to the molecules Reaper, Hid and Grim, which bind to and inhibit a IAP in *D. melanogaster* (Salvesen et al. 2002).

Omi/HtrA2 is a serine protease, which is phylogenetically strongly conserved with a homologue in bacteria named HtrA2. The protease activity is dependent on a strongly conserved Serine Protease Motif and trimerization mediated by a Trimerization Motif (Li et al. 2002). XIAP, cIAP1 and cIAP2 have been shown in several instances to be substrates for proteolytic cleavage through Omi/HtrA2 (Yang et al. 2003; Suzuki et al. 2004). A recent study demonstrated that the mature serine protease Omi/HtrA2 can degrade XIAP, accounting for an additional mechanism by which this protein inhibits IAPs (Srinivasula et al. 2003). Also, through its serine protease activity, Omi/HtrA2 can activate caspase-9 independently of the apoptosome, and it also exerts a caspase-independent pro-apoptotic activity.

p53

The tumor suppressor p53 conveys diverse signals upon DNA-damage eliciting a variety of effects including cell-cycle arrest, apoptosis, and senescence (Vousden et al. 2002). p53 has been known for more than 25 years and, due to its central role in the response to genome damage, it has been termed "the guardian of the genome". This

molecule was included in the present work due to its prominent position upstream of the intrinsic apoptotic pathway (Vousden et al. 2002). However, a detailed review and analysis of all features of this protein would be beyond the scope of the present work. It is important to note that p53 mainly functions as a transcription factor. For this purpose it carries sequences necessary for shuttling the molecule across the nuclear membrane, such as the nuclear export signal, a DNA-binding domain, and a transactivation domain. In addition, to exert its transcription factor function p53 assembles into homotetramers. This is mediated by an oligomerization domain. The regulation of this protein predominantly occurs through posttranslational modifications (Bode et al. 2004). A large number of modifications involving specific residues have been reported, including phosphorylation at Ser $^{6, 9, 15, 20, 37, 46, 149, 315, 376, 378, 392}$ and at Thr $^{18, 55, 81, 150, 155}$, acetylation at Lys $^{305, 320, 382}$, neddylation at Lys $^{370, 372, 373}$, and sumoylation at Lys ^{386}.
An additional feature worth to be mentioned is the occurrence, in human p53, of a polymorphism at codon 72. The occurrence of Arg instead of Pro at this location is related to poor prognosis in some cancers although it is associated with an increased apoptotic potential (Dumont et al. 2003).

5. Materials and Methods

Cell lines and cultures

The MDCK normal epithelial cell line which is derived from a Cocker Spaniel was obtained from the American Type Culture Collection (Rockville, Maryland 20852, USA). It was grown in Iscove's Mod. Dulbecco's Medium (Sigma, St. Louis, USA) supplemented with 10% fetal calf serum inactivated at 60°C, 2.5% HEPES buffer (Sigma, St. Louis, USA), 1% L-Glutamine (Sigma, St. Louis, USA) and 1% Penicillin-Streptomycin solution (Sigma, St. Louis, USA).
The canine normal keratinocyte line used was previously generated from a Beagle dog as described (Kolly et al. 2005). The cells were grown in Dulbeccos' Modified Eagles Medium (Sigma, St. Louis, USA) supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin and 15% fetal calf serum. All cell culture reagents were obtained from Gibco BRL life Sciences (Basel, Switzerland). Both cell lines were grown at 37°C and a 5% CO_2 atmosphere.

RNA extraction and RT-PCR

Total RNA was isolated from MDCK cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). In addition, RNA was harvested from normal canine keratinocytes for those molecules where cDNA amplification from MDCK cells failed (most likely due to a low expression level of the targeted molecule) or if it appeared necessary to confirm the sequence obtained. All RNA extractions were done according to the manufacturer's protocols.

Subsequently, 40 U Protector RNAse Inhibitor (Roche, Mannheim, Germany) were added to each RNA extract. cDNA was synthesized from 1µg of total RNA using the 1st strand cDNA Synthesis Kit for RT-PCR (AMV) and an oligo-dT-primer according to the manufacturer's instructions (Roche, Mannheim, Germany).

Primers including the putative Start and Stop codons as deducted from publicly available sequence information and taking the human sequence as a reference (Tables 1 and 2) were designed either manually or by using the Primer3 website (http:// frodo.wi.mit.edu/) (Rozen et al. 2000). Molecules to be amplified were chosen based on their relative importance in the pathway and in order to provide a representative set of the pathway. Reverse transcription PCR reactions were accomplished with 1µl of cDNA samples and Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). Basic cycling parameters were as follows: initial denaturation at 94 °C for 1.5 min; 35 cycles of denaturation at 94 °C for 50 sec, annealing for 50 sec at 50-70 °C (a gradient was chosen on dependance of the primer melting temperature) and extension at 72 °C for a time adjusted to the expected size of the product (1 min per 1000 bp); final extension for 10 min at 72 °C. Each PCR reaction was optimized by adjusting the annealing temperature and the final magnesium concentration to yield a band with the size corresponding to the expected principal transcript for each molecule. After agarose gel separation, the amplificates were recovered and purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sent to a company (Microsynth AG, CH-9436 Balgach) for direct sequencing (Cycle Sequencing / Capillary Electrophoresis) with the primers used for amplification.

Table 1: Primer sequences

Molecule	Up-primer	Dn-primer
Bad	5`-AGAGC**ATG**TTCCAGATCCC-3`	5`-GGACGCGGGACGGG**TCA**C-3`
Bak	5`-GAGAA**ATG**GCATCCGGGC-3`	5`-CCTCTGGGAG**TCA**TGATTTG-3`
Bax	5`-TG**ATG**GACGGGTCCGGGG-3`	5`-GGCC**TCA**GCCCATCTTTTTC-3`
Bcl-w	5`-GG**ATG**GCGACCCCAGCC-3`	5`-GC**TCA**CTTGCTCGCAAAAAAG-3`
Bcl-XL	5`-CCATCCCTATTATAAAA**ATG**TCTCA-3`	5`-GGGTAAGTGGGTGGTCAGTG-3` *
Caspase-3	5`-CC**ATG**GAGAACACTGAAAACTC-3	5`-CCTCACTTGGCATATGAACC-3` *
Caspase-8 isof. B	5`-TGACCAAC**ATG**GATTTCAGC-3`	5`-CA**TCA**ATTAGGAGGGAAGAAGAG-3`
Caspase-9 alpha	5`-CC**ATG**GACGAGGCGGAGC-3`	5`-GGTGAGGGGCCTCA**TTA**C-3`
cIAP-1	5`-AGAGGACA**ATG**GAGGATAG-3`	5`-TATAGCTGACTT**TTA**TGAGAG-3`
cIAP-2 var. 1	5`-CT**ATG**AACATAGTACAAAATAGC-3`	5`-GTCTTGGGTCTTT**TCA**GG-3`
Mcl-1	5`-AGGAGCTTGCG**ATG**TTCGG-3`	5`-CACTTAAAAGG**CTA**TCTTATTAG-3`
Noxa	5`-AG**ATG**CCCGGCCGGAAG-3`	5`-A**TCA**AGGTTCCTGAGCGGAAG-3`
Omi/HtrA2 var. 1	5`-CTG**ATG**GCTGCACTGAGG-3`	5`-CCCAC**TCA**TTCTGTGACTTC-3`
p53	5`-CTGCG**ATG**CAAGAGCCAC-3`	5`-GAAG**TCA**GTCTGAGTCGGG-3`
Smac/DIABLO alpha	5`-GGCCGTCACAGG**ATG**GCGG-3`	5`-GCGC**TCA**ATCCTCACGCAG-3`
Survivin	5`-**ATG**GGCGCTTCGTCGCTG-3`	5`-GAG**CTA**TTCTGCGGCGGC-3`
XIAP	5`-AAGAGAAG**ATG**ACTTTTAACAG-3`	5`-CTGTAGAGTGAGA**TTA**AGAC-3`

Bold: Start and Stop codons; * Stop codon is upstream of primer sequence

Cloning

If the amplification product corresponded to the expected sequence, the PCR was repeated using primers containing restriction enzyme sites suitable for cloning into the GST-tagged pGEX-4T2 vector (Invitrogen, Carlsbad, USA). Amplicons were cloned and the vectors were transformed into DH5 alpha competent cells. Miniprep plasmid DNA was sequenced using the pGEX 5'- and 3'-sequencing primers (5'-GGGCTG GCAAGCCACGTTTGGTG-3' and 5'-CCGGGAGCTGCATGTGTCAGAGG-3'). Final cDNA sequences were derived from the consensus of at least three individual clones and were deposited in GenBank.

5'-RACE

For Bad and XIAP a 5'-RACE was performed with the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. The PCRs were done using the Universal Primer A Mix provided with the Kit and with primers BadGSPDn2 (5'-CTGCTGGTGC TTGCCAGGGCTTG-3') and XIAPGSPDn (5'-GCACCGCACAGTATCTCCTTCA CCA-3'). In both cases, the recommended touch-down PCR protocol was applied and an additional control PCR with primer pairs spanning two exons (BadGSPUp/Bad GSPDn [5'-AGCAGGAAGACTCCAGCCCTGCAAA-3' / 5'-AGCTGTGGCGACTCCGGGTC TCAG-3'] and XIAPGSPUp/XIAPGSPDn2 [5'-CATACCCGGAGAACCCTGCCAT GTA-3' / 5'-CC AAGGGTCTTCACTGGGCTTCCAA-3']) was performed.

PCR products were separated by agarose gel electrophoresis and recovered with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). These products were cloned into the pCR2.1-TOPO vector provided with the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) and sent out for sequencing (Microsynth AG, CH-9436 Balgach) using the M13 forward and reverse primers.

Computational analysis

Putative canine protein sequences were derived from the cDNA sequences using a translation tool, accessible via the ExPASy homepage (http://us.expasy.org/). Canine nucleotide coding sequences and deducted protein sequences were compared with human and murine counterparts from GenBank (Table 2) using the EMBOSS sequence analysis tool provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align/). The nucleotide and the protein sequences were aligned with the needle method and the Blosum62 matrix algorithm, respectively.

The exon/intron structure was derived by aligning the cds with the respective genome sequence using BLAT (Blast like alignment tool) on the MGC genome browser (http://mgc.ucsc.edu/) (Kent 2002). The genomic sequence release date was May 2005 for canines, October 2005 for murines, and March 2006 for humans. Presence of canonical splice sites and identity of the diverse canine coding sequences obtained with the canine genome sequence were further verified using Spidey, a mRNA-to-genomic alignment program available at NCBI (http://www.ncbi.nlm.nih.gov/).

For domain identity/similarity analyses the human domain signatures available from UniProtKB/Swiss-Prot and additional relevant features as suggested from the current literature were used.

Table 2: GenBank nucleotide and protein accession numbers

Molecule	Human	Mouse	Canine
Bad	NM_004322, NP_004313	NM_007522, NP_031548	DQ127247, AAZ32936 *
Bak	NM_001188, NP_001179	NM_007523, NP_031549	DQ002813, AAY19401 *
Bax var. alpha	NM_138761, NP_620116	NM_007527, NP_031553	NM_001003011, NP_001003011
Bcl-2 isof. alpha	NM_000633, NP_000624	NM_009741, NP_033871	AB154172, BAD05044
			AY509563, AAR92491
Bcl-w	NM_004050, NP_004041	NM_007537, NP_031563	DQ116955, AAZ22484 *
Bcl-XL	NM_138578, NP_612815	NM_009743, NP_033873	NM_001003072, NP_001003072
Caspase-3	NM_004346, NP_004337	NM_009810, NP_033940	NM_001003042, NP_001003042
Caspase-8 isof. B	NM_033355, NP_203519	NM_009812, NP_033942	DQ223013, ABB02414 *
Caspase-9 alpha	NM_001229, NP_001220	NM_015733, NP_056548	DQ116956, AAZ22485 *
cIAP-1	NM_001166, NP_001157	NM_007465, NP_031491	DQ223014, ABB02415 *
cIAP-2 var. 1	NM_001165, NP_001156	NM_007464, NP_031490	EF102104, ABL09004 *
Mcl-1	NM_021960, NP_068779	NM_008562, NP_032588	NM_001003016, NP_001003016
Noxa	NM_021127, NP_066950	NM_021451, NP_067426	DQ449072, ABE02691 *
Omi/HtrA2 var.1	NM_013247, NP_037379	NM_019752, NP_062726	DQ138643, AAZ66770 *
p53	NM_000546, NP_000537	NM_011640, NP_035770	AF060514, AAC16909
			AB020761, BAA78379
Smac/DIABLO alpha	NM_019887, NP_063940	NM_023232, NP_075721	EF088670, ABK96902 *
Survivin	NM_001168, NP_001159	NM_009689, NP_033819	AB180206, BAD20570
			AY741504, AAU89275
			AB095108, BAC22748
XIAP	NM_001167, NP_001158	NM_009688, NP_033818	DQ225116, ABB03778 *

*: Own submissions (generated through the present work)

6. Results

6.1 Novel canine apoptosis-associated cDNAs (coding sequences)

The complete coding sequence (cds) of eleven novel canine apoptosis-associated molecules belonging to the Bcl-2 family, the Caspases, the Inhibitors of Apoptosis Proteins and their mitochondrial inhibitors was identified on th canine genome sequence and cloned. Clones were derived from MDCK cells except when otherwise stated. The putative genomic structure spanning the cds of these molecules was determined by aligning the own sequences with the second dog genome sequence release (CanFam2.0) from May 2005 available at NCBI. In the following, basic data for each of these cDNAs are presented, together with a comparison to the human and murine counterparts (Table 3). Alignment of the canine sequences generated in this study with the dog genome sequence showed 100% identity and canonical splice sites in all cases except where stated otherwise. Where appropriate, reference is made to the genome sequence-based GNOMON automated predictions available at NCBI. All novel canine sequences generated have been deposited in GenBank under the accession numbers indicated in Table 2.

*Bcl-w (*BCL2L2*)*

The cds of the canine anti-apoptotic Bcl-2 family member Bcl-w is 582 bp long and consists of two exons, which is exactly the same for the human and murine cds. The identity of the canine Bcl-w cds with its human counterpart is 95%, while the identity between human and mouse is 94%.

*Bak (*BAK1*)*

The cds of the canine multi-domain pro-apoptotic Bcl-2 family member Bak consists, like its human counterpart, of 636 bp distributed over five exons. The murine cds is nine basepairs shorter (627 bp). The human sequence has a higher similarity with the canine than with the murine sequence (90% and 80% identity, respectively).

Bad

The cds of the canine BH3-only Bcl-2 family member Bad consists of three exons, spanning 504 bp in length. Its sequence is three bp shorter than the human cds and 111

bp shorter than the murine cds, which spans over four exons. Since several gaps occur in the alignment between human and canine cds, additional clones were generated from normal canine keratinocytes to exclude a MDCK-related artefact. Clones from both cell types yielded the same sequence.

In addition, a 5'-RACE was performed because the GNOMON prediction available at NCBI [GenBank: XM_540884] suggested that the canine Bad transcript and ensuing protein are longer at the 5'-end. The analysis performed using mRNA from normal keratinocytes revealed a 5'-UTR of 90 bp for Bad, alignment of the sequence obtained with the dog genome predicts an additional proximal exon consisting of 82 bp. This additional sequence shows no similarity with the GNOMON prediction.

The canine Bad cds is 86% identical to its human counterpart, whereas the identity between human and mouse is only 65%, due to the additional coding exon at the 5'-end in the murine sequence. When only overlapping parts are compared, identity between human and mouse sequences is 82%.

*Noxa (*PMAIP1*)*

The cds of the canine BH3-only Bcl-2 family member Noxa consists, like its human counterpart, of two exons with a total of 165 bp. The murine cds is divided into three exons, spanning 312 bp. The canine Noxa cds was derived from normal keratinocytes. It yields 87% identity with the human sequence. In contrast, the mouse sequence differs remarkably from the human sequence (40% identity), which ensues from the presence of an additional coding exon at the 5'-end in the murine sequence. Comparison of overlapping parts only yields 73% identity between human and mouse sequences.

Caspase-8

The cds of canine Caspase-8 consists of eight exons with a total of 1461 bp. It shares 82% identity to its human counterpart, Caspase-8 isoform B. Both human and murine cds are shorter than the canine counterpart (1440 bp and 1443 bp, respectively). The degree of identity of the murine Caspase-8 sequence with the human sequence (76%) is similar to that of the canine molecule (82%).

Caspase-9

The cloned canine Caspase-9 cds is 1338 bp long, is distributed over nine exons, and is a homologue of the human Caspase-9 variant alpha. Interspecies comparison revealed 77% identity between canine and human sequences which is similar to the 76% identity observed between mouse and human sequences. The canine sequence generated displayed two single nucleotide mismatches as compared to the dog genome reference sequence (bp 261 G instead of T; bp 269 G instead of C). The second mismatch results in an amino acid substitution (Arg instead of Thr). It should be noted that the two canine spliced ESTs available for this region so far [GenBank: DN439319 and DN401609] support a G at position 269.

*cIAP-1 (*BIRC2*)*

The cds of the canine member of the Inhibitors of Apoptosis Proteins (IAP) family cIAP-1 (BIRC2) consists of 1794 bp and eight exons. It is 63 bp shorter than the human cds and 45 bp shorter than the murine cds. Comparison with the human cIAP-1 cds revealed 82% identity, which is slightly less than the 84% identity between human and mouse sequences. Alignment of the complete mouse coding sequence on the mouse genome sequence is not possible because of a gap in this region.

*cIAP-2 (*BIRC3*)*

The cds of the canine IAP-family member cIAP-2 (BIRC3) is 1815 bp long and spans over eight exons. There is one mismatch in the nucleotide sequence (bp 1242 G instead of A) as compared to the dog genome sequence which has no effect on the cIAP-2 amino acid sequence. Accordingly, the canine cds generated is 99% identical with the GNOMON cIAP-2 variant 1 prediction [GenBank: XM_546551]. Like the mouse sequence, the canine cIAP-2 sequence shares 76% identity with the human sequence.

*XIAP (*BIRC4*)*

The cds of the canine IAP-family member XIAP (BIRC4) has 1482 bp in total and is separated into six exons. A 5'-RACE was performed because the GNOMON prediction available at NCBI [GenBank: XM_538165] suggested that the canine XIAP transcript and protein are longer at the 5'-end. The analysis performed using mRNA isolated from normal keratinocytes revealed a 5'-UTR of 64 bp for XIAP and an additional upstream

exon spanning over 32 bp. Splicing of this exon corresponds to the predicted splicing. The data indicate a shorter mRNA as predicted by GNOMON. A previously reported partial cds [GenBank: AY603038, bp 1090 to 1482], derived from canine liver tissue, is 100% identical with the sequence generated in this study.
The canine and murine sequences show a similar degree of homology with the human counterpart (90% and 89% identity, respectively).

Omi/HtrA2

As in humans and mice, the cds of the canine mitochondrial inhibitor of IAPs Omi/HtrA2 is 1377 bp long and divided into eight exons. The sequence obtained in this study displayed a single non-coding nucleotide mismatch as compared to the dog genome reference sequence (bp 927 T instead of A). Homology of the canine Omi/HtrA2 cds with the human counterpart is higher than that of the murine cds (91% vs. 86% identity, respectively).

Smac/DIABLO

The cds of canine Smac/DIABLO, another mitochondrial inhibitor of IAPs, consists, like the mouse cds, of 714 bp separated into six exons. The human cds is six bp longer. The canine cds has a higher homology with human Smac/DIABLO than the mouse cds (90% vs. 84% identity, respectively).

Table 3. Nucleotide and protein sequence comparison of human, murine and canine apoptosis-associated molecules

Molecule	Number of coding exons			Coding sequence similarity			Protein length (aa)			Protein sequence: identity (similarity)			
	Hu	Mo	Ca	Hu / Mo	Hu / Ca	Ca / Mo	Hu	Mo	Ca	Hu / Mo	Hu / Ca	Ca / Mo	
Bcl-2 [AB154172]	2	2	?	88%	89%	83%	239	236	239	89% (90%)	89% (90%)	84% (85%)	Bcl-2 family
Bcl-2 [AY509563]			?		89%	93%			236		89% (90%)	94% (96%)	
Bcl-w	2	2	2	94%	95%	93%	193	193	193	98% (99%)	100%	99% (99%)	
Bcl-XL	2	2	2	94%	97%	93%	233	233	233	98% (99%)	100%	98% (99%)	
Mcl-1	3	3	3	77%	89%	76%	350	331	350	76% (83%)	89% (93%)	77% (83%)	
Bak	5	5	5	80%	90%	78%	211	208	211	76% (85%)	92% (95%)	75% (86%)	
Bax var. alpha	6	6	?	90%	93%	89%	192	192	192	92% (94%)	97% (98%)	93% (95%)	
Bad	3	4	3	65%	86%	64%	168	204	167	60% (66%)	80% (83%)	63% (69%)	
Noxa	2	3	2	40%	87%	40%	54	103	54	34% (38%)	83% (91%)	32% (39%)	
Caspase-3	6	6	6	84%	89%	83%	277	277	277	87% (94%)	89% (93%)	86% (93%)	Caspases
Caspase-8 isof. B	8	8	8	76%	82%	74%	479	480	486	67% (78%)	76% (86%)	66% (81%)	
Caspase-9 alpha	9	9	9	76%	77%	79%	416	454	445	72% (78%)	72% (79%)	73% (82%)	
cIAP-1	8	?	8	84%	82%	77%	618	612	597	83% (91%)	87% (92%)	80% (88%)	IAPs
cIAP-2 var.1	8	8	8	76%	76%	71%	604	600	604	74% (85%)	75% (85%)	69% (81%)	
XIAP	6	6	6	89%	90%	86%	497	496	493	87% (93%)	87% (92%)	84% (90%)	
Survivin	4	4	4	81%	89%	80%	142	140	142	82% (91%)	91% (96%)	83% (92%)	
Omi/HtrA2 var. 1	8	8	8	86%	91%	85%	458	458	458	84% (89%)	90% (92%)	85% (88%)	Others
Smac/DIABLO alpha	6	6	6	84%	90%	86%	239	237	237	85% (91%)	90% (95%)	87% (94%)	
p53	10	10	10	78%	81%	76%	393	390	381	76% (82%)	81% (87%)	72% (80%)	

Hu: human; Ca: canine; Mo: mouse; ?: Numbers of exons not determined due to gap in genome sequence; [ ]: GenBank accession no. of canine sequence (all data shown in the table were derived using dog sequences generated in this study with exception of the 2 cases with accession numbers indicated in square brackets)

6.2 Previously described canine apoptosis-associated cDNAs

The cds of six canine apoptosis-associated molecules whose mRNA sequences had been already previously deposited in GenBank by others were identified on the dog genome sequence and cloned. Basic data derived from the own cDNA clones are handled and reported here similarly as in the preceding section 6.1 and compared with the human and murine sequences (Table 3). Differences between precedent submissions, the own sequences and/or the canine genome sequence are pointed out. Clones were derived from MDCK cells except where otherwise stated. Again, alignment of the canine sequences generated in this study with the dog genome sequence showed 100% identity and canonical splice sites except where otherwise stated.

Bcl-2 isoform alpha

Attempts to amplify the cds of the canine anti-apoptotic Bcl-2 molecule from both MDCK and normal keratinocytes RNA using several primer combinations [not shown] failed possibly due to low expression levels of Bcl-2 in these cell types. However, due to the importance of this molecules within the pathway, for further analysis the two canine Bcl-2 sequences previously submitted to GenBank by others were used, i. e. the provisional complete mRNA reference sequence [GenBank:AB154172] and a further submission restricted to the cds [GenBank:AY509563]. The former sequence was derived from peripheral mononuclear blood cells of an adult dog, the origin of the latter was not disclosed. Both cds only share 85% identity with each other. Alignment of both sequences with the dog genome reference sequence revealed a gap of approx. 100 bp in the latter within the first exon. Homology between the two GenBank sequences is lowest in this region. The cds of AB154172 shows higher homology with the dog genome sequence than AY509563 (99% vs. 95% identity, respectively, by a coverage of 85%).

Comparison with human Bcl-2 isoform alpha cds revealed 89% identity for both canine sequences which is close to the figure observed between human and murine sequences (88%).

*Bcl-XL (*BCL2L1*)*

A complete mRNA sequence for the canine anti-apoptotic Bcl-2 family member Bcl-XL is available under GenBank: NM_001003072. It was derived from lymphocytes of an

adult dog. All clones generated during the present work had a single non-coding nucleotide substitution (C instead of T) at position 666 of the cds, compared to the previously submitted sequence. The dog genome reference sequence supports a C at this position. In all three species investigated the cds has the same length and is distributed over two exons, the canine sequence shows a slightly higher homology with the one of humans than with the one of mice (97% and 94%, respectively).

Mcl-1

A complete mRNA reference sequence for the canine anti-apoptotic Bcl-2 family member Mcl-1 is available under GenBank: NM_001003016. It was derived from canine lymphocytes. Comparison of this sequence with the Mcl-1 cds generated in this study shows four single mismatches and two gaps. Alignment of the own sequence with the dog genome sequence indicated one single non-coding substitution at position 436 (C instead of T). Alignment of the reference sequence with the dog genome revealed a single bp insert at position 31 and a gap 9 bp further on. In addition, there were five single mismatches at positions 6 (T instead of C), 119 (G instead of A), 436 (C instead of T), 641 (A instead of G) and 978 (C instead of T) of the cds. Two of these mismatches lead each to an amino acid substitution (position 119: Arg instead of Lys; position 641: Gln instead of Arg), the latter involving the BH3-domain. The insert at bp 31 leads to a short frameshift resulting in a change of amino acids 11-13 (ArgThrGln instead of GlyLeuAsn); this region does not code for any known domain.
In both humans and dogs the Mcl-1 cds is 1053 bp long, in contrast to 996 bp in mice. In all three species investigated the cds is distributed over three exons. The canine cds shares a higher homology with the human counterpart than the murine sequence (89% and 77% identity, respectively).

Bax

An mRNA sequence comprising the complete cds of the canine multi-domain pro-apoptotic molecule Bax is available under GenBank: NM_001003011. It was derived from an osteosarcoma cell line. The sequence generated in this study is 100% identical to the GenBank sequence. Alignment with the dog genome sequence showed that a large portion of the 3'-end is not covered by the genome sequence precluding determination of the number of coding exons. In all three species investigated the cds is 579 bp

long, in mice and humans it is distributed over six exons. The cds of both dogs and mice show a similar degree of homology with the human Bax var. alpha (93% and 90% identity, respectively).

In addition, an alternative splice form of the canine Bax cds was detected, which is similar to the human Bax gamma version [GenBank: NM_138762]. Like its human counterpart, the cds of canine Bax gamma consists of 126 bp divided into two exons. It shares 91% identity with the human Bax gamma cds. *In silico* translation predicts, like in humans, a hypothetical protein of 41 amino acids (identity human/canine: 83%).

Caspase-3

A complete canine Caspase-3 mRNA reference sequence derived from dog lymphocytes is available under GenBank: NM_001003042. Comparison of this sequence with the Caspase-3 cds from this study shows two single non-coding nucleotide substitutions at position 126 (T instead of A) and 756 of the cds (A instead of T). The dog genome sequence supports an A and a T, respectively, at these positions.

In all three species investigated the cds is 834 bp long and is distributed over six exons, the canine sequence has greater homology with the human counterpart than with the murine sequence (89% vs. 84% identity).

*Survivin (*BIRC5*)*

Three mRNA sequences are available from GenBank for canine Survivin (BIRC5). The first complete mRNA entry was derived from the T-cell lymphoma line CL-1 [GenBank: AB095108]. The next complete mRNA entry from dog testis is available as the reference sequence under GenBank: AB180206. A third mRNA sequence available is of unknown origin [GenBank: AY741504].

The own cds is 100% identical with the second and third sequence (except for a N at position 272 of AY741504), whereas it only shows 91% identity with the first submisson, due to 26 single nucleotide mismatches and ten gaps at the 3'-end. The first submission yields surprising 97% identity with the human cds. However, it shows less than 95% identity with the dog genome sequence, while the own sequence aligns with 100% identity.

Like the human Survivin isoform 1, all four canine cds span over 429 bp in total, while the murine cds is six bp shorter. In all three species the cds is distributed over four

exons. The own canine cds shows greater homology with the human counterpart than the murine sequence (89% vs. 81% identity).

*p53 (*TP53*)*

Two complete canine p53 cds are available in GenBank (AB020761 and AF060514). The first was derived out of spleen tissue and the second, which is the provisional reference sequence, from peripheral blood leucocytes. The own sequence is 100% identical to the first sequence and aligns to 100% with the dog genome sequence. A gap is present in the canine genome sequence between the first and second coding exon. In contrast, the second sequence shows 99% similarity to the dog genome due to seven mismatches, leading to the exchange of three amino acids (position 2: Glu instead of Gln; position 4: Ser instead of Pro; position 378: Leu instead of Pro).

With 1146 bp, canines have the shortest cds compared to humans and mice (1182 bp and 1173 bp, respectively), but in all three species the cds is distributed over ten exons. The dog sequence shares a slightly higher homology with the human counterpart than the murine sequence (81% vs. 78% identity).

6.3 Sequence comparison of canine apoptosis-associated proteins with the human and murine orthologues

In this part of the study all available canine apoptosis-associated protein sequences as deduced from the own cDNA data presented in the preceding sections (and, in the case of Bcl-2 from the two previous GenBank submissions by others) were analyzed *in silico*. The degree of conservation between humans, canines and mice of the whole proteins as well as of specific domains, motifs and residues most relevant to apoptosis (chosen according to the human domain signatures available at UniProtKB/Swiss-Prot and from selected references) was determined. Data are reported in detail in Tables 3, 4, 5, and Figures 2 and 3.

Bcl-2 family

The canine aa sequences of four anti-apoptotic (Bcl-2, Bcl-w, Bcl-XL and Mcl-1), two multi-domain pro-apoptotic (Bak and Bax) and two BH3-only family members (Bad

and Noxa) were available (Tables 3 and 4). The overall identity of Bcl-2 family proteins ranged from 80% to 100% between dogs and humans, and from 34% to 99% between mice and humans. In all cases there was a higher degree of homology between human and canine sequences than between human and murine sequences, except for Bcl-2, where dogs and mice showed the same degree of similarity to the human counterpart. The length of the Bcl-2 family proteins is relatively constant in canines compared to humans, while it markedly differs for some murine proteins, in particular the BH3-only members Bad and Noxa (Table 3).

The anti-apoptotic family members are highly conserved, with two molecules, Bcl-XL and Bcl-w, 100% identical at the amino acid level between humans and dogs. In mice, three out of 15 BH domains analyzed in this subgroup differ from the human counterpart by one aa, while in dogs this only occurs with one BH domain (belonging to one out of the two canine versions of Bcl-2 [GenBank: AY509563]). The transmembrane domains show either 100% conservation in all three species or slight differences with the human counterpart in both the murine and canine versions.

The multi-domain pro-apoptotic members Bak and Bax show a slightly lower degree of interspecies conservation than the anti-apoptotic members. In the mouse, four out of the six BH domains of these two molecules showed one amino acid exchange, the remaining two BH domains showed two. In the dog only two out of these six BH domains show one amino acid exchange. The transmembrane domain of Bax is 100% conserved across the three species, that of Bak shows a change of four amino acids in mice and of two amino acids in dogs.

The lowest overall and domain identity in the Bcl-2 family was observed with the two BH3-only proteins Bad and Noxa. This is particularly true for the murine molecules, which are longer than the canine and human counterparts, and display each one additional coding exon, thus resulting in low identity percentages. Comparison of overlapping regions of these proteins between murines and humans yielded 76% and 64% identity for Bad and Noxa, respectively. In addition, murine Noxa displays a second BH3 domain (Oda et al. 2000) absent from both the human and canine counterparts. However, regulatory serine residues (Ser $^{75, 99, 118, 134}$ in humans) required for maintaining Bad in an inactive state and bound to cytosolic 14-3-3Sigma proteins (Bae et al. 2001) are conserved among the three species at nearly the same locations.

Several human Bcl-2 family molecules exhibit Caspase-3 substrate recognition motifs (Figure 2). One out of two Caspase-3 substrate recognition motifs in Mcl-1 (Michels et al. 2004) is not conserved in dogs and mice, the Caspase-3 substrate recognition motifs in Bcl-2 varies in dependance of the version in the dog, but both motifs in Bcl-XL are conserved in all three species (Clem et al. 1998; Bellows et al. 2000). The Caspase-3 substrate recognition motif in human pro-apoptotic Bad is conserved in dogs and mice, while an additional motif reported in mice (Condorelli et al. 2001) is absent from the human and canine proteins. The caspase substrate recognition motif in human pro-apoptotic Bax (Wood et al. 1998) is conserved in the other two species.

Table 4. Degrees of identity (%) of human, canine and murine Bcl-2 family members at the domain level

	BH1		**BH2**		**BH3**		**BH4**		**TM**	
Molecule	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca
Bcl-2	100	100	100	100	93*	100	100	100	91	95
Bcl-w	100	100	100	100	100	100	100	100	100	100
Bcl-XL	100	100	94*	100	100	100	100	100	100	100
Mcl-1	95	100	100	100	100	100	-	-	100	100
Bak	90*	100	94	100	93*	93*	100	100	78	89
Bax	95*	95*	87	100	93*	100	100	100	100	100
Bad	-	-	-	-	87	87	-	-	-	-
Noxa	-	-	-	-	73	87	-	-	-	-

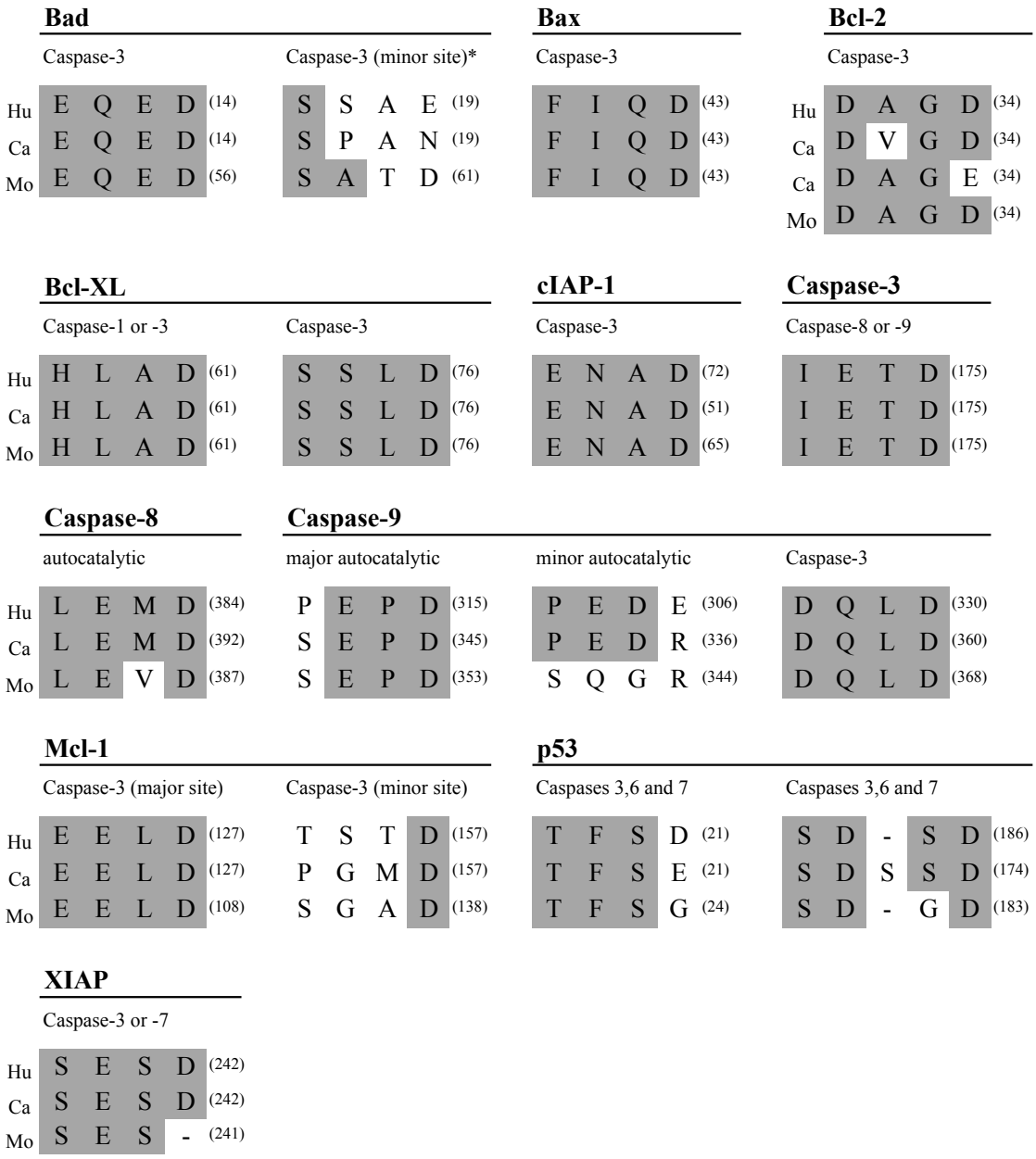
BH: Bcl-2 Homology domain (numbered from 1 to 4); TM: Transmembrane domain; Hu: human; Ca: canine; Mo: mouse; *: 100% similarity; -: domain not present;
respective domain length (number of amino acids): BH1=20, BH2=16, BH3=15, BH4=21, $TM_{(Bcl-2)}$=22, $TM_{(Bcl-XL)}$=17, $TM_{(Mcl-1,Bax)}$=21, $TM_{(Bak)}$=18

Caspases

The sequences of initiator Caspase-8 and -9 and effector Caspase-3 were available for comparison. The protein length is equal in all three species for Caspase-3 and varies little for Caspase-8 (isoform B). Mice and dogs have a much longer Caspase-9 alpha amino acid sequence than humans (Table 3). Compared to dog and mouse there is a gap of 32 and 35 aa, respectively, in the human sequence immediately C-terminal of the CARD domain. The corresponding sequences in dogs and mice consist of additional nucleotides within the body of exon two and show a relatively low degree of homology at the amino acid level (57%) between each other.

The degree of the overall sequence and domain homology of both Caspase-3 and Caspase-9 alpha between humans, dogs and mice is very high. In contrast, murine Caspase-8 differs more strongly from the human sequence than the canine version (67% and 76% identity, respectively). In particular, the degree of identity of both death effector domains between human and canine compared to one between human and murine differs by up to 20%. Dogs retain all 37 residues conserved across all human DEDs (Barnhart et al. 2003), while in mice two of these are substituted by similar amino acids. The sequences of the active sites of these three caspases are 100% identical in all species analyzed. Most caspase cleavage sites within these molecules are either identical or present with slight, likely not functionally significant differences (Figure 2). The sole exception is a minor autocatalytic recognition motif identified *in vitro* in human Caspase-9 (Srinivasula et al. 2001), which appears to be restricted to this species. An IAP-binding motif occurring at the N-terminal end of the small subunit of human Caspase-9 is well conserved in dogs and mice (Figure 3). A motif responsible for interaction of Caspase-3 with the linker N-terminal of the BIR2 of XIAP (Riedl et al. 2001) shows slight variations between the species examined ($SGVD^{179}$ in humans, $SGIE^{179}$ in dogs, $SGTD^{179}$ in mice). The residue of Caspase-3 interacting with BIR2, Met^{182} in humans (Riedl et al. 2001; Scott et al. 2005), is conserved in the other two species.

Figure 2. Interspecies conservation of caspase substrate recognition motifs in the apoptosis-associated molecules investigated



Bold: substrate; plain: specific caspase; Hu: human, Ca: canine, Mo: mouse; numbers in brakets indicate position of last amino acid; recognition motifs of Bcl-2: second row: canine sequence [GenBank: BAD05044], third row: canine sequence [GenBank: AAR92491]; residues in grey are identical to human sequence; -: gap in alignment; *: minor Caspase-3 substrate recognition motif described in murine Bad

Inhibitors of Apoptosis Proteins (IAPs)

The sequences of four canine members of the IAP family (cIAP-1, cIAP-2, XIAP, and Survivin) were available for comparison with their human and murine counterparts (Table 3 and 5). These IAPs show a rather constant protein length across the species, except for canine cIAP-1, which is 21 amino acids shorter than the human and murine counterparts. As a whole, this family of molecules shows a similar degree of homology between the three species. In some instances and for some of the domains (in particular the BIRs of cIAP2 and XIAP), the murine proteins are slightly more closely related to the human counterparts than the canine molecules (Table 5).

Findings on cIAP-1, cIAP-2 and XIAP are presented together because of the structural similarity of these molecules. The RING-type motif is generally highly conserved (close to 100% identical to human) in all three canine and murine molecules (Table 5). The CARD domains of cIAP-1 and cIAP-2 show a higher degree of homology to the human counterpart in dogs than in mice (Table 5). A Caspase-3 substrate recognition motif found in human cIAP-1 (Clem et al. 2001) is conserved in dogs and mice (Figure 2). A Caspase-3 and Caspase-7 substrate recognition motif described in human XIAP (Deveraux et al. 1999) is conserved in dogs but not in mice (Figure 2). Individual residues involved in the diverse functions of the BIR domains such as Tyr^{154} and Phe^{228} of BIR2 of human XIAP and the linker residues N-terminal to the BIR2 (human $DISD^{151}$) involved in the interaction with caspases are present in all three species investigated (Riedl et al. 2001; Scott et al. 2005).

Canine Survivin is, like the human conterpart, 142 amino acids long and exhibits a higher overall and BIR domain sequence homology than the murine protein. Interaction with Caspase-9 has been associated with phosphorylation at Thr^{34} (O'Connor et al. 2000), and this residue is conserved in both dogs and mice.

Table 5. Degrees of identity (%) of human, canine and murine IAP family members at the domain level

	cIAP-1		cIAP-2 var.1		XIAP		Survivin	
	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca	Hu/Mo	Hu/Ca
BIR1	88	94	88	85	93	96	-	-
BIR2	94	96	82	76	97	93	-	-
BIR3	90	96*	84	82	92	86	92	97*
CARD	89	96*	63	69	-	-	-	-
RING-type	94*	97*	97*	97*	92	94*	-	-

BIR: Baculovirus Inhibitor of Apoptosis Repeat (numbered from 1 to 3); CARD: Caspase Recruitment Domain; Hu: human; Ca: canine; Mo: mouse; *: 100% similarity; -: domain not present; respective domain length (number of amino acids): BIR1=68, BIR2=67, $BIR2_{(XIAP)}$=68, BIR3=68, $BIR3_{(XIAP)}$=66, $BIR_{(Survivin)}$=71 (Survivin's BIR most closely resembles to the BIR3 of other molecules)

Mitochondrial inhibitors of IAPs Smac/DIABLO and Omi/HtrA2

Both murine and canine Smac/DIABLO proteins are two amino acids shorter than human Smac/DIABLO version alpha (Table 3). Dogs show a higher overall homology and a higher homology of the Mitochondrial Transition Domain (respective identities: 85% and 73%) and of the Diablo Homolog region (respective identities: 91% and 89%) with the human Smac/DIABLO sequence than mice. The IAP-binding Motif (IBM) constituting the N-terminus of the mature protein ($AVPI^{59}$ in the immature human protein) is 100% identical in all three species.

Omi/HtrA2 proteins display the same length in all three species (Table 3). The overall sequence identity with the human counterpart is higher for dogs than for mice. The canine mitochondrial targeting domain has a slightly lower homology with the human counterpart than the murine sequence (68% vs. 74%), while the transmembrane domain presents with a clearly higher degree of homology in canines than in murines (95% vs. 81%). The Serine Protease Motif is highly conserved across mice, dogs and humans. The Trimerization Motif ($QYNFIA^{151}$ in humans) is identical in the three species. In contrast, and despite the otherwise high degree of homology between human and canine Omi/HtrA2, the region corresponding to the IBM in canine Omi/HtrA2 ($SVLG^{137}$) significantly differs from the human and murine sequences (Figure 3).

Figure 3. IAP-binding motif (IBM) of selected proteins from different species

Smac/DIABLO*	A	V	P	I
HuCaspase 9	A	T	P	F
CaCaspase 9	A	V	P	F
MoCaspase 9	A	V	P	Y
HuOmi/HtrA2	A	V	P	S
CaOmi/HtrA2	**S**	**V**	**L**	**G**
MoOmi/HtrA2	A	V	P	A
DrReaper	A	V	A	F
DrHid	A	V	P	F
DrSickle	A	I	P	F

Hu: human; Ca: canine; Mo: mouse; Dr: *D. melanogaster*; *: motif is identical in Hu, Ca and Mo; residues in grey are shared by at least 50% of the proteins listed. The motif indicated for canine Omi/HtrA2 was identified basing on the full-length alignment with the human orthologue.

p53

The canine p53 protein shares higher overall identity with the human p53 (81%) than mice (76%), but it is shorter than in the two other species. The DNA-binding domain and the nuclear export signal are slightly better conserved in mice (96% and 100% identity, respectively) than in dogs (95% and 92% identity, respectively). In contrast, the oligomerization domain and the transcription activation domain are better conserved in canines (97% and 84% identity, respectively) than in mice (94% and 75% identity, respectively).

In dogs six phosphorylation sites (corresponding to human Ser $^{9, 37, 46}$ and Thr $^{55, 81, 150}$) are not conserved at the exact same location in the alignment and for three of these residues (corresponding to human Ser ^{9} and Thr $^{55, 150}$) there are no potential alternative residues in the vicinity. Similarly, in mice six of these sites (corresponding to human Ser $^{37, 46, 149}$ and Thr $^{55, 81, 155}$) are not conserved at the same location in the alignment. All other post-translational modification sites mentioned in chapter 4 are conserved in the three species.

The region surrounding codon 72 in humans (APR^{72}VA) was examined. A gap of eight amino acids is present in the dog-to-human sequence alignment in this region, while the sequence is completely different in mice (PAAQD^{71}).

Two non-classical Caspase-3, -6 and -7 substrate recognition motifs described in humans (Sayan et al. 2006) appear not to be conserved in dogs, while only one out of the two is conserved in mice (Figure 2).

7. Discussion

In this study, the cDNAs of a representative set of canine apoptosis-associated molecules were cloned and analyzed. The results allow for making inferences about the reliability of publicly available canine sequences such as the dog genome sequence and the Genbank mRNA entries. The second release of the dog genome estimated a coverage of 99% with an error rate less than 10^{-4} (Lindblad-Toh et al. 2005). Although gaps were found in the genome sequence within three of 18 analyzed genes, data of the present study indicate that, in general, the currently available genome sequence is accurate since only six mismatches per 16,611 bp of sequence cloned were found. These mismatches may not uniquely derive from sequencing errors but could at least in part represent single nucleotide polymorphisms, since the dog genome data and the sequences of the present study were derived from different dog breeds. Further possible reasons for discrepancies include somatic cell variation and RNA editing. The second release of the dog genome (CanFam2.0) from May 2005 is significantly improved compared to the first release, where a larger number of mismatches were found upon comparison with the sequences generated in this study. Those mismatches included in particular indels in the alignments with the Caspase-9 and cIAP-1 sequences from this study, which resulted in frame shifts in the automated GNOMON predictions ([GenBank: XM_544557.1, XM_536600.1], data not shown). Based on the genes analyzed in this study GNOMON predictions for canine proteins appear relatively reliable. They contained all novel cds generated in this study with the exception of Noxa. However, the automated program predicted an additional sequence at the 5'-end of two molecules (Bad and XIAP) that was not supported by the 5'-RACE data from this study.

The same general remarks apply for the limited number of mismatches observed between the cDNA clones generated in this study and mRNA sequences previously submitted to Genbank by other research groups. In the majority of these cases, however, the cDNA sequences of this study were consistent with the dog genome sequence. A

recent investigation involving analysis of approximately 30,000 non-redundant human mRNA sequences suggested that mRNA collections may contain a substantial number of errors (Furey et al. 2004).

The sequence data generated in this study allowed to perform a thorough interspecies comparative analysis at the nucleotide and protein level for a representative number of molecules of the intrinsic apoptotic pathway. In general and with a few notable exceptions, a higher homology was observed between human and canine sequences than between human and murine sequences. This is consistent with a faster rate of mutation that has been observed in the mouse as compared to dogs and humans (Lindblad-Toh et al. 2005).

In the following, the findings of the analysis for the main families of each molecule examined are discussed.

Bcl-2 family

A gradient in the degree of interspecies conservation was observed among the subgroups depending on the number of BH domains contained. The anti-apoptotic molecules showed the highest level of overall and domain interspecies conservation, with two members, Bcl-XL and Bcl-w, conserved to 100% at the protein level between humans and dogs. This high conservation might be explained by the fact that several domains (i. e. BH1, BH2 and BH3) of these molecules participate in forming a functional structure calling for very specific sterical requirements (Cory et al. 2002).

In contrast, the BH3-only subgroup showed the lowest degree of interspecies homology within the Bcl-2 family. They exert their function mainly through one single domain, which is compatible with the lower overall degree of conservation observed. A closer similarity between canine and human molecules than between murine and human sequences becomes particularly evident from the analysis of BH3-only molecules Bad and Noxa. Both molecules significantly diverge in mice, especially outside of the BH3 domain (Oda et al. 2000). Recent studies have revealed that the anti-apoptotic members of the Bcl-2 family each bear a unique pattern of interaction with peptides derived from BH3 domains of BH3-only molecules, indicating that conservation of this domain may be particularly important (Certo et al. 2006). The strong degree of conservation of the dog Bcl-2 proteins suggests, at least from a mechanistical point of view, that this

species is potentially well suited for modeling interactions between Bcl-2 family members and therapeutic interventions based on BH3 peptides. Successful use of such reagents in a preclinical setting has been reported in several instances (Goldsmith et al. 2006; Perez-Galan et al. 2007).

Caspases and specific substrate recognition motifs

The high degree of conservation of the active sites as well as of autologous and heterologous substrate recognition motifs in the caspase sequences analysed in the present study indicate that the basic mechanisms are well conserved across species, and further confirmed by the high degree of phylogenetic conservation of these molecules (Shi 2002). The analysis of the present study revealed the presence of an identical minor sequence difference of a major autocatalytic substrate recognition motif of human Caspase-9 in dogs and mice. This difference has been previously shown not to impair autocatalytic cleavage of the murine proenzyme but it lead to more efficient *in vitro* cleavage by Caspase-8 as observed with the human orthologue (McDonnell et al. 2003), thus suggesting potential functional differences at this level of the pathway between humans and other species. Data of the present work suggest that the canine molecule would behave similarly as the murine protein. Furthermore, this finding suggests that cleavage of Caspase-9 might be a functionally important process although it does not appear to be necessary for autoactivation (Stennicke et al. 1999). In contrast, a minor autocatalytic substrate recognition motif reported for human Caspase-9 (Srinivasula et al. 2001) is absent both in the dog and mouse. This finding might be explained by the proposed minor importance of this motif in humans.

The analysis performed in this study shows that the cleavage site of Caspase-3 is conserved in the three species investigated. This is expected, since cleavage is crucial for the activation of the effector caspases. Several of the apoptosis-associated molecules examined in this study contain Caspase-3 substrate recognition motifs. In most molecules bearing one single recognition motif, its sequence is conserved in the three species, suggesting that the corresponding cleavage sites are indeed of physiological importance. The recognition motif of XIAP constitutes a notable exception. Due to its absence in rat, murine and bovine XIAP, this motif was previously considered to be unique to the human molecule (Hell et al. 2003). Results from this study show that the

motif is conserved in canine XIAP, which constitutes a specific similarity between dogs and humans.

In the case of molecules yielding multiple caspase substrate recognition motifs, the extent of their conservation in canines and/or mice is mostly compatible with the respective classification of the corresponding cleavage sites into either major or minor, as previously shown for Mcl-1 (Michels et al. 2004). The case of p53 is particular insofar as the two caspase substrate recognition motifs described for the human protein are non-classical. This study data suggest that the two caspase substrate recognition motifs of p53 might both be absent from the canine proteins, which needs to be corroborated by functional studies. If this finding could be confirmed, it might represent a biologically significant difference between humans and dogs, since it has been shown that the cleavage products of human p53 are localized in the mitochondria where they appear to exert a pro-apoptotic function (Sayan et al. 2006).

Inhibitors of Apoptosis Proteins (IAPs)

Results of this study show an overall high degree of conservation of the BIR domains of the molecules analyzed and of the functionally significant residues participating in the interactions with caspases in all three species investigated, supporting their functional importance and, likely, that their biological function is strongly dependent on sterical requirements. The same applies for further motifs, such as the RING-type motif of cIAP-1, cIAP-2 and XIAP, and the less well characterized CARD domains of cIAP-1 and cIAP-2. Collectively, the strong conservation of all domains examined in this family, despite their large size, is remarkable.

Mitochondrial inhibitors of IAPs

The analysis in this study revealed that while the IBM of Smac/DIABLO is 100% identical in humans, dogs and mice, surprisingly, the IBM of Omi/HtrA2 is not conserved in dogs, since the tetrapeptide sequence AVPS (in humans) is replaced, most likely, with SVLG. The relevant sequences of the novel Omi/HtrA2 clones generated in this study are fully supported by the dog genome sequence. Interestingly, the dog shares a high sequence similarity in this region with cattle as deduced from ESTs (Li et al. 2002) and from the sequence of a GNOMON predicted bovine homologue of Omi/HtrA2 [GenBank: XP_601332.1, data not shown]. A N-terminal alanine has been

repeatedly shown to be required for binding of IBMs to IAPs (Suzuki et al. 2001; Li et al. 2002) with a few exceptions. A N-terminal serine residue occurs in Caspase-8 processed human Caspase-7 allowing Caspase-7 binding to the BIR-2 of XIAP (Scott et al. 2005). Moreover, a recent study has revealed the existence, in humans, of further mitochondrial proteins with potential IBM-mediated, IAP antagonistic function (Verhagen et al. 2006). While the majority of them displayed a N-terminal alanine, one protein (glutamate dehydrogenase) has a N-terminal serine residue essential for interaction with XIAP. In addition, studies using phage display have revealed peptides bearing an N-terminal serine capable of binding BIR-2 of XIAP or BIR-3 of cIAP1 (Franklin et al. 2003; Kurakin et al. 2004). These findings suggest that the sequence differences between the human and the putative canine Omi/HtrA2 IBM observed in this study could represent different IAP binding spectrums in the two species and in different effects at the functional level. In addition, XIAP, cIAP1 and cIAP2 have been shown in several instances to be substrates for proteolytic cleavage through Omi/HtrA2 (Yang et al. 2003; Suzuki et al. 2004), and binding through the IBM appears to potentiate this effect (Yang et al. 2003). In some experimental settings, however, Omi/HtrA2's IBM appears dispensable for cell killing activity (Li et al. 2002). Collectively, these findings from the literature and from this study raise questions about the importance of binding of IAPs through the IBM motif of Omi/HtrA2 for apoptosis in mammals. The differences in this motif between canines and humans could be significant with respect to tumorigenesis or development of resistance to cancer therapy. Therefore, further investigations on the mode of function of canine Omi/HtrA2 are needed.

p53

The central, upstream location of this molecule implies that specific interspecies differences might have impacts on several molecular pathways including cancerogenesis. Due to the great variety and extreme complexity of the processes in which this molecule is involved, the present analysis was restricted to a few important domains and particular features of p53. The results showed that most post-transcriptional modification sites known in humans are conserved in dogs and mice, although some differences were observed. An example is the lack of a threonine, corresponding to human Thr^{55}, in both dogs and mice. Human p53 has been shown to be

phosphorylated at this residue by TAF 1, which leads to its Mdm2-mediated degradation (Li et al. 2004). In response to DNA damage, phosphorylation at Thr^{55} through TAF1 is reduced and p53 is stabilized. This rises the question how this and other known differences in the posttranslational modification of p53 affect the downstream pathway functions in dogs and mice.

In addition, interestingly, both dogs and mice display significant sequence differences, compared to humans, in the region of codon 72. These sequence variations are likely to have a significant impact on mechanisms related to tumorigenesis in these species. Codon 72 presents with a specific polymorphism in humans, where the occurrence of Arg^{72} instead of Pro^{72} is related to poor prognosis in some cancers although it is associated with an increased apoptotic potential (Dumont et al. 2003). Further support for the notion that this polymorphism and the associated characteristics are a peculiarity of humans comes from a recent study, which identified a proline at this location and the absence of polymorphism in non-human primate species (Puente et al. 2006).

8. Conclusions

In summary, a comparative interspecies analysis of a representative set of canine apoptosis-associated molecules related to the intrinsic pathway was performed in this study. On one side, the search unveiled a generally high degree of similarity of canine apoptosis-associated molecules as compared to the human counterparts, thus supporting the notion that spontaneous canine cancer may represent, at least on a mechanistical basis, adequate models of the human disease. For example, the high degree of conservation of the canine Bcl-2 family members and domains suggests that dogs might be well suited for modeling therapies targeting the BH domains. On the other hand, the comparative analysis revealed a small number of sequence peculiarities potentially significant at the functional level within canine molecules associated with the highly conserved intrinsic apoptotic pathway. These variations include: (1) the lack of conservation of the putative IBM of the canine mitochondrial protein Omi/HtrA2, and (2) the absence of single caspase substrate recognition motifs in apoptosis-associated molecules; (3) the absence of some post-translational modification sites documented in

the human p53 protein. In addition, it was found that canine XIAP yields a caspase substrate recognition motif previously considered to be unique to humans.

The molecules analyzed in this study will assist further investigations, which will be needed to determine the evolutionary significance and the functional impact of these differences in both normal and cancer cells. In addition, taking advantage of the wealth of information now available from the dog genome sequence it should be possible to easily identify new molecules related to cancer related pathways and include them in future analyses. Such efforts will enhance the evaluation of spontaneous canine cancers for their value as models of human cancer.

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